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TOXOPLASMOSIS IN CAPTIVE DOLPHINS (*TURSIOPS TRUNCATUS*) AND WALRUS (*ODOBENUS ROSMARUS*)

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Abstract

Toxoplasma gondii infection in marine mammals is intriguing and indicative of contamination of the ocean environment and coastal waters with oocysts. *Toxoplasma gondii* infection was detected in captive marine mammals at a sea aquarium in Canada. Antibodies to *T. gondii* were found in all 7 bottlenose dolphins (*Tursiops truncatus*) tested. Two of these dolphins, as well as a walrus (*Odobenus rosmarus*) at the facility, died. Encephalitis and *T. gondii* tissue cysts were identified in histological sections of the brain of 1 dolphin (dolphin no. 1). Another dolphin (dolphin no. 2) had mild focal encephalitis without visible organisms, but viable *T. gondii* was isolated by bioassay in mice and cats from its brain and skeletal muscle; this strain was designated TgDoCA1. The PCR-RFLP typing using 11 markers (B1, SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico) identified a Type II strain. The DNA sequencing of B1 and SAG1 alleles amplified from TgDoCA1 and directly from the brains of dolphin no. 1 and the walrus showed archetypal alleles consistent with infection by a Type II strain. No unique polymorphisms were detected. This is apparently the first report of isolation of *T. gondii* from a marine mammal in Canada.

Toxoplasma gondii infections are widely prevalent in humans and other animals worldwide (Dubey and Beattie, 1988). Numerous studies reported the existence of *T. gondii* infections in marine mammals, including sea otters, dolphins, seals, and whales (Cole et al., 2000; Lindsay, Thomas et al., 2001; Miller et al., 2001; Dubey et al., 2003; Miller et al., 2004; Thomas et al., 2007). A toxoplasmosis-like illness was reported in Atlantic bottlenose dolphins (*Tursiops truncatus*) from the United States (Migaki et al., 1977; Cruickshank et al., 1990; Inskeep et al., 1990; Schulman et al., 1997; Dubey et al., 2003), and Italy (Di Guardo, Agrimi et al., 1995; Di Guardo, Corradi et al., 1995). Serologic surveys indicated a

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very high prevalence of antibodies to *T. gondii* in free-range *T. truncatus* from both coasts of the United States (Dubey et al., 2003, 2005). Viable *T. gondii* was recently isolated from 3 free-range and beached *T. truncatus* (Dubey et al., 2008).

MATERIALS AND METHODS

Naturally infected dolphins and walrus

The marine mammals referred to were maintained at a captive facility in Canada. The pinnipeds and dolphins were housed in 2 different buildings. There were 8 bottlenose dolphins, 7 Pacific walruses (*Odobenus rosmarus*), 10 California sea lions (*Zalophus californianus*), 7 harbor seals (*Phoca vitulina*), and 2 gray seals (*Halichoerus grypus*) housed in different pools or buildings. The water in these buildings does not intermix, but animals are occasionally exchanged. Artificial salt water is made by adding sodium chloride with no other mineral additions; chlorine and ozone are added for disinfection. The pinniped facilities have chlorine levels maintained at low levels compared to the dolphin chlorine levels, which is related to the sensitivity of the pinniped eyes to higher levels of chlorine. Additional water quality parameters include salinity between 1.75 and 2.5%, pH between 7.5 and 7.6, and water temperature approximately 22 C.

The pinnipeds and dolphins are both fed a diet consisting primarily of 2 species of fish, i.e., herring and capelin. This is fresh fish caught once a year (capelin, spring), or twice a year (herring, spring and fall). The fish are caught by commercial fishermen off the coast of New-foundland, Canada, and immediately placed on ice, stored and inspected at the factory, and frozen. Fish is shipped on pallets by way of freezer trucks, and inspected at the sea aquarium on arrival. Fish is thawed overnight by water and coolers, and fed to the animals the next day. Excess fish is not reused for pinnipeds or cetaceans. In addition, the animals receive daily vitamin supplements in the form of commercially available marine mammal vitamins. The animals that died were necropsied at the sea aquarium. For *T. gondii* tests, serum, tissues, or both, from the dead animals (Table I) were shipped overnight on ice to the Animal Parasitic Diseases Laboratory (APDL), U.S. Department of Agriculture, Beltsville, Maryland.

Serological examination

Serum samples were tested for *T. gondii* antibodies with the use of dilutions from 1:25 to 1:3,200 with the modified agglutination test (MAT) as described by Dubey and Desmonts (1987).

Bioassay for *Toxoplasma gondii*

Fresh, unfrozen brain and skeletal muscle from dolphin no. 2 were received at APDL on 13 April 2007 (3 days after death). Samples (50 g) of brain and skeletal muscle were homogenized separately, digested in acid–pepsin (Dubey, 1998), and processed for inoculation into 5–15 out-bred female Swiss Webster (SW) mice obtained from Taconic Farms, Germantown, New York, as described by Dubey et al. (2002).

For bioassay in cats, samples of brain and muscle were fed separately to 2 *T. gondii*–free cats (Dubey et al., 2002). One cat was fed approximately 50 g of brain over a period of 24 hr and the other car was fed approximately 500 g of muscle over a period of 3 days. Feces of cats were examined for shedding of *T. gondii* oocysts 3–24 days after feeding on the dolphin tissues. Fecal floats were incubated in 2% sulfuric acid for 1 wk at room temperature on a shaker to allow sporulation of oocysts, and then were bioassayed by oral administration to SW mice (Dubey and Beattie, 1988).

Inoculated mice were examined for *T. gondii* infection. Tissue imprints of lungs and brains of mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on day 41 postinoculation (PI) and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies with the MAT. Mice were killed 43 days PI, and brains of all mice were examined for tissue cysts as described (Dubey and Beattie, 1988). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in tissues.

Genetic characterization

Toxoplasma gondii DNA was extracted from infected tissues of the dolphins and the walrus, or from mice that were inoculated with *T. gondii*-infected tissues from 1 of the dolphins. Strain typing was performed with the use of the genetic markers B1, SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico (Miller et al., 2004; Dubey et al., 2006; Su et al., 2006).

Immunohistochemical examination

Samples of all major organs (brain, gastric compartments, spleen, heart, intestine, liver, kidney, lungs, mesenteric lymph nodes, adrenal glands, testis, eye, and pancreas) were examined and fixed in 10% buffered formalin. Tissues were processed routinely for paraffin embedding and sectioning. After staining with a hematoxylin–phloxine–saffron stain (HPS) or periodic acid Schiff reaction after counterstaining with hematoxylin stain (PAS), sections were examined microscopically.

Paraffin embedded sections of brains of 2 dolphins (Numbers 1 and 2; Table I) and the walrus were sent to the APDL for immunohistochemical staining. At APDL, paraffin embedded tissue sections were reacted with antibodies to *T. gondii* and *Neospora caninum* polyclonal rabbit antibodies as described (Lindsay and Dubey, 1989; Dubey et al., 2001).

RESULTS

Upon microscopic examination, dolphin no. 1 was found to possess rare protozoan tissue cysts scattered throughout the brain parenchyma (Fig. 1). The tissue cysts were thin walled (<0.5 μm thick) and up to 60 μm in diameter; they contained numerous PAS positive bradyzoites. Some tissue cysts were surrounded by glial nodules; there was evidence of necrosis (Fig. 1). Rare dense clusters of mononuclear cells were present within the meninges. The tissue exhibited diffuse, moderate cerebral edema as revealed by a moderate number of hyaline droplets diffusely present in most Virchow Robin spaces. In the meninges, there was focal infiltration by a moderately dense population of mononuclear

cells composed in decreasing order of plasma cells, lymphocytes, and macrophages. In the fasciculata layer of the adrenal cortex, there were many randomly distributed, often confluent foci of lytic necrosis. In the cells bordering the necrotic foci, there were single, variably sized although generally large, amphophilic intranuclear inclusion bodies, usually surrounded by a clear halo and marginated chromatin. These were interpreted as consistent with Herpes virus infection. The tissue cysts reacted positively to *T. gondii* antibodies, but not to *N. caninum* (Fig. 1B). Tachyzoites were not observed.

In dolphin no. 2, microscopic examination of the brain showed rare, randomly distributed glial nodules, and a single cuff of lymphocytes around a single medium sized vein within the meninges covering the parietal lobe. The brain of the walrus showed moderate to severe, diffuse, nonsuppurative meningitis. No protozoan organisms were seen in histological sections from the walrus or dolphin no. 2.

Antibodies to *T. gondii* were found in all dolphins tested at the sea aquarium (Table I). *Toxoplasma gondii* was isolated from dolphin no. 2 by bioassay in cats and mice. The 2 cats that were fed brain and muscle shed oocysts; *T. gondii* tachyzoites were also found in tissues of mice that were fed oocysts. The 4 mice inoculated with the brain of dolphin no. 2 developed antibodies to *T. gondii* in their serum 31 days PI, and tissue cysts were found in their brains when killed day 48 PI. The mice inoculated with the muscle tissue of dolphin no. 2 developed neither antibodies to *T. gondii* nor tissue cysts in their brains. The *T. gondii* isolate from dolphin no. 2 was designated TgDoCA1.

The DNA extracted from each of the 4 mice inoculated with the brain of the dolphin and 1 mouse that was fed oocysts from the cat fed muscle of the dolphin was Type II by PCR-RFLP at all loci except Apico, which was Type I. DNA sequencing at 2 polymorphic loci (B1 and SAG1) identified only canonical Type II or III alleles. Type II and III strains share the identical allele at B1 and SAG1. According to the PCR-RFLP data, dolphin no. 2 was infected with a Type II strain.

DNA sequencing of the B1 and SAG1 PCR products amplified directly from formalin-fixed sections of the brain of the walrus and dolphin no. 1 also identified archetypal Type II or III alleles. Altogether, the data are consistent with infection of a Type II genotype in all 3 marine mammals at this sea aquarium facility in Canada.

DISCUSSION

Results of genotying on 3 marine mammals kept at a Canadian sea aquarium indicated that a Type II strain had infected the dolphins and the walrus. Recently, *T. gondii* was isolated from 3 free-ranging bottlenose dolphins (TgDoUs1-3) from the east coast of the United States (from a stranded group of dolphins). The TgDoUs1 isolate was also Type II, except at locus c22-8, whereas TgDoUs2 and TgDoUs3 were clonal Type II (Dubey et al., 2008). Similarly, the *T. gondii* isolate from a striped dolphin (*Stenella coeruleoalba*) in the Pacific Coast of Costa Rica was a Type II strain (Dubey et al., 2007). The *T. gondii* isolates from other marine animals have been either Type II, or Type X and A, which are prevalent in the Pacific coastal marine environment (Conrad et al., 2005; Sundar et al., 2008). Thus, neither

Type I nor Type III isolates have so far been detected in marine mammals. Taken together, it is clear that the Type II lineage predominates not only in the North American and European continents, but also in the marine environment.

Results of serological examination indicated that all dolphins had been exposed to *T. gondii* infection. The magnitude of antibody titer was not indicative of the timing of parasite recruitment because the MAT only detects IgG antibodies, and dolphin no. 2 had a persistently high titer (1:12,800) at least for 6 mo. Although viable *T. gondii* was obtained from the brain and muscle of that dolphin, histologically there was no evidence of the parasites in this animal even though there were rare, randomly distributed, glial nodules. The apparent detection of *T. gondii* DNA in the brain of the walrus, despite a negative MAT at 1: 25 serum dilution, is of note because it may represent a recently acquired infection without sufficient time for IgG antibodies to have developed.

The present study is apparently the first isolation of viable *T. gondii* from a marine mammal in Canada. Mikaelian et al. (2000) reported toxoplasmosis in Beluga whales from St. Lawrence estuary, Quebec, Canada; *T. gondii* was found in histological sections of tissues of 2 animals and MAT antibodies were found in 6 of 27 whales. Measures et al. (2004) found antibodies in 9% of 122 grey seals (*Halichoerus grypus*), 9% of 34 harbor seals (*Phoca vitulina*), and 2% of 60 hooded seals (*Cystophora cristata*) from the east coast of Canada. Antibodies to *T. gondii* were reported in 3 of 53 walruses from Alaska, United States (Dubey et al., 2003). The ingestion of oocysts in contaminated food or water and the ingestion of *T. gondii*–infected tissues are the 2 main sources of postnatal *T. gondii* infection. The mechanism of *T. gondii* infection in marine mammals is most intriguing because their main staple diet includes fish or invertebrates, i.e., cold-blooded animals, or they are exclusively herbivorous. Thus, ingestion of *T. gondii*–infected meat is unlikely. *Toxoplasma gondii* infection of dolphins is even more intriguing because they drink little or no water; their water requirements are satisfied by consumption of fish, squid, or other cold-blooded sea animals (Elsner, 1999). Accidental ingestion of infected carcasses of birds or rodents, or of water contaminated with cat feces, may occur. This route, however, probably does not play a significant role in the high seropositivity in marine mammals. Miller et al. (2002) suggested that land-based surface runoff was a significant risk for *T. gondii* infection in sea otters, because the *T. gondii* oocysts could be washed into the sea via runoff contaminated by cat excrement. *Toxoplasma gondii* does not parasitize any cold-blooded vertebrates. However, molluscs (a major food source for dolphins) can filter large quantities of water and, consequently, concentrate *T. gondii* oocysts. This idea is supported by experimental data showing that *T. gondii* oocysts can be concentrated by oysters and mussels (Lindsay, Phelps et al., 2001; Arkush et al., 2003).

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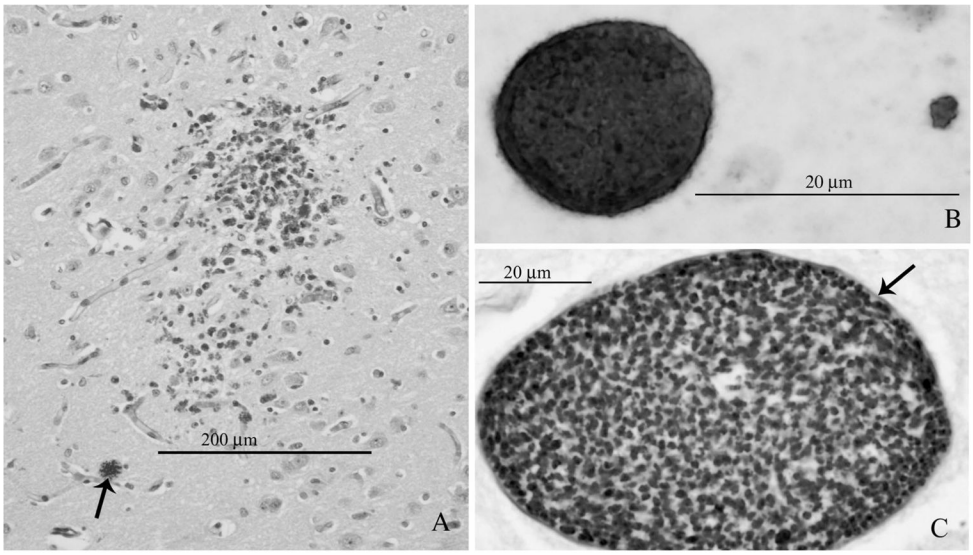

Figure 1.

Lesions and *Toxoplasma gondii* in the brain of dolphin no. 1 (Tulo). (**A**) Loose nodular aggregate of predominant macrophages and microglia in a necrotic focus of the neuropil. Note adjacent PAS-positive tissue cyst (arrow). PAS. (**B**) Two groups of *T. gondii* reacted with polyclonal *T. gondii* antibodies. Immunohistochemical staining with polyclonal *T. gondii* rabbit antibodies. PAS and hematoxylin. (**C**) A tissue cyst stained with PAS. Note thin (arrow) tissue cyst wall.

Table I

Summary of marine mammals with antibodies to *Toxoplasma gondii*.*

Species/identifier	Age (yr), sex	MAT titer	Time in captivity (yr)	Origin	Clinical signs	Cause of death (if applicable)
Dolphin no. 1	20, M	12,800	6	Russia	Disorientation, anorexia	Toxoplasmosis-induced meningoencephalitis
Dolphin no. 2	15, M	12,800	5.5	Russia	Lethargy, anorexia	Open/unknown
Walrus no. 1	2, F	<25	<1	Russia	Terminal seizure	Cause undetermined†
Dolphin no. 3	15, F	51,200	8	Russia	NA	Alive
Dolphin no. 4	12, F	400	8	Russia	NA	Alive
Dolphin no. 5	15, F	12,800	7	Russia	NA	Alive
Dolphin no. 6	18, F	51,200	8	Russia	NA	Alive
Dolphin no. 7	12, F	400	7	Russia	NA	Alive
Dolphin no. 8	3, F	50	3	Captive-born at same facility	Anorexia, keratitis, kidney failure,	Open/unknown

*F = female, M = male, NA = not applicable.

†*Toxoplasma gondii* DNA demonstrated in the brain by PCR.